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Inactivation of Substance P by Cultured Human Endothelial Cells

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Vasoactive peptides that are released or transported in blood come immediately in contact with vascular endothelium. In the past, endothelium was considered merely a diffusion barrier to the passage of substances across vessel walls. More recently it was recognized that endothelium is a complex tissue that is intimately involved in homeostatic mechanisms, such as the metabolism of vasoactive peptides (2,5). Because of the development of techniques for growing endothelial cells in culture (6,7,12), it is now possible to investigate the metabolism of peptides by isolated human vascular endothelial cells. We found previously that kinins and angiotensins are cleaved by endothelial cells (9,10). The present investigation extends these studies to include the metabolism of Substance P (SP) by human endothelial cells.

MATERIALS AND METHODS

Endothelial cells were collected by perfusing human umbilical cord veins with sterile trypsin solution (9,10). The cells were grown in monolayer cultures in medium 199 containing 20% fetal calf serum and 10% human serum. A confluent monolayer was obtained within 7 to 10 days. The cells were transferred or used for experiments when they reached 70 to 80% confluency. Cultured cells were suspended from monolayers by brief treatment with 0.25% trypsin. The suspended cells were collected into medium containing 10% heated serum to stop the action of trypsin, were centrifuged to a pellet, and were washed at least three times with 10 ml of protein-free medium before using them in an experiment. For comparison, in some experiments the cells were removed from the monolayers by scraping with a rubber spatula and were then washed as described.[1]

Initially, the number of cells in each experiment was determined by counting aliquots of cell suspension in a hemocytometer. The number of viable cells was established by excluding those that stained with trypan blue (0.5%). To avoid errors caused by clumping of cells during the suspension and subsequent washing procedures, the number of cells was also determined by measuring the DNA content of each cell suspension. The techniques used for identification of endothelial cells and the methods for

[1] These scraped cells were tested directly for the inactivation of SP and also after incubation with trypsin (0.25%) for 15 min at 37°C.

measurement of the content of protein and DNA were given previously (10).[2]

SP and its octapeptide derivative, SP_8, were obtained from commercial sources. The octapeptide derivative contained the 4 to 11 C-terminal amino acid sequence of the undecapeptide and was approximately half as active as SP in stimulating contraction of isolated strips of guinea pig ileum. Inactivation of SP and the octapeptide was measured by bioassay on atropinized guinea pig ileum (13). Concentrations of the peptides that stimulated submaximal contractions were used to establish a dose-response relationship. Then the spasmogenic response to peptides that had been incubated with endothelial cells or cell extracts was measured. Inactivation of the peptides by the cells was determined by following the decrease in the smooth muscle stimulating activity with the time of incubation.

When intact cell monolayers were used as a source of the enzyme, the cultures with the cells on the bottom of 250 ml flasks were washed three times with 10 ml of fresh, protein-free culture medium. Then the peptides to be tested as substrates were added directly to the culture flasks in protein-free culture medium. The flasks were agitated gently on a rocker platform that was placed inside the CO_2 incubator at 37°C. Samples for bioassay were withdrawn at 0 time and at regular intervals for 5 to 60 min and placed in an ice bath. All samples were assayed within 5 to 15 min after collection.

Suspended endothelial cells or cell lysates were tested for inactivation of SP of SP_8 in an incubation mixture that contained the peptide substrate (10^{-6} M), 0.2 M Tris buffer (pH 7.4), and 0.2 M NaCl. When inhibitors were used, they were added to the cells in Tris buffer 15 min prior to addition of the peptide substrate. The samples were incubated at 37°C after addition of either inhibitors or substrates. For some experiments the endothelial cells were lysed by either repeated freezing and thawing, or by N_2 cavitation (16).

RESULTS

Endothelial cells in tissue culture inactivated both SP and the octapeptide, SP_8. Intact, undisturbed monolayers of cells cleaved the peptides at a slower rate than did the suspended or lysed cells (Table 1). The rate of inactivation of SP was approximately the same regardless of the method used for detaching the cells. Thus, either treatment with trypsin or scraping of the cells yielded preparations that cleaved about 44 to 50 nmole SP per hr.

As with the angiotensin I converting enzyme (10), the SP inactivating enzyme is probably on the surface of the cells. This was indicated by the fact that there was no increase in enzymatic activity when the cells were disrupted by freezing and thawing, or by N_2 cavitation (Table 1). However

[2] In order to determine whether trypsin used for detaching the cells could be bound by endothelial cells and could contribute to their enzymatic activities, we measured the uptake of ^{125}I-labeled trypsin. The cells were detached from monolayers with 0.25% trypsin containing 10^6 cpm/ml. They were washed in serum-containing medium and then at least three times in serum-free medium as described. The amount of radioactivity in the washes and final cell pellet was measured in a gamma radiation counter.

TABLE 1. *Inactivation of SP and the SP_8 octapeptide by cultured human endothelial cells*

Cell preparation	Treatment	No.	Substrate and Conc.(M)		Activity nmole/hr/10^6 Cells
Monolayer	---	3	SP	2×10^{-6}	3.4 ± 0.7
"	---	3	octapeptide	2×10^{-6}	1.8 ± 0.2
Suspended cells	trypsin (0.25%)	7	SP	10^{-6}	49 ± 10
"	scraped	6	"	"	60 ± 14
Lysed cells	frozen-thawed	5	"	"	47 ± 14
"	N_2 cavitation	3	"	"	44 ± 6
Suspended cells	trypsin (0.25%)	5	octapeptide	"	40 ± 10

Cells were incubated at 37°F and pH 7.4 with substrates. Samples assayed on guinea pig ileum.

TABLE 2. *Inhibition of the enzyme(s) in endothelial cells that inactivate SP*

Inhibitor	Conc	Substrate	No.	% Inhibition
Soybean trypsin	600 μg/ml	SP (10^{-6}M)	6	88
Trasylol*	1,000 u/ml	"	4	85
o-phenanthrolene	10^{-3}M	"	6	0
Leucyl-methionine amide	10^{-4}M	"	4	41
TAMe	10^{-3}M	"	5	63
DFP	10^{-3}M	"	4	83

in contrast to the effect on the angiotensin I converting enzyme (10), freezing and thawing of the cells did not decrease activity of the SP inactivating enzyme.[3]

Various inhibitors were used in an attempt to characterize the enzymes that cleaved SP and the octapeptide. The results of experiments with enzyme inhibitors of the inactivation of SP are summarized in Table 2. Typical experiments in which inactivation of SP was measured both with and without inhibitors are shown in Figs. 1 and 2. Of the several enzyme inhibitors tested, *o*-phenanthrolene, a metal chelating agent that inhibits peptidases,

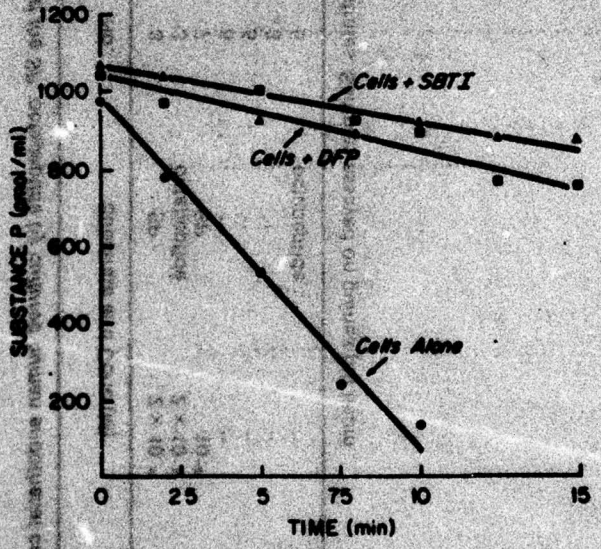

FIG. 1. Inactivation of SP by endothelial cells. Suspended cells (0.9×10^6) were incubated with buffer alone or with soybean trypsin inhibitor (SBTI) or di-isopropylfluorophosphate (DFP) for 15 min at 37°C. Concentrations of inhibitors given in Table 2. SP was added (10^{-6}M) and the activity measured at 0 to 10 min by bioassay on guinea pig ileum. The amount of peptide remaining in the reaction mixture is given on the ordinate and time on the abscissa.

*When labeled trypsin was used to detach endothelial cells, approximately 1% of the label (equivalent to 2.5 μg of trypsin) was retained by the cells. It was not determined whether this enzyme remained active after it was associated with the cells. However, in other experiments, cells first scraped from the monolayer and then incubated with trypsin were not more active in cleaving SP than cells scraped and only washed.

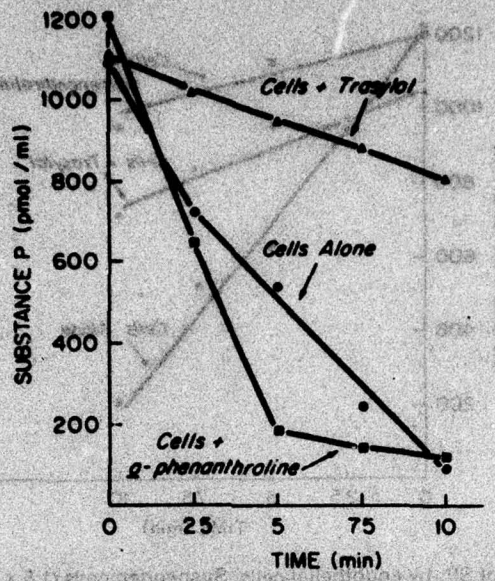

FIG. 2. Inactivation of SP by endothelial cells. Suspended cells (1.5×10^5) were incubated with buffer alone or with Trasylol® or o-phenanthroline for 15 min at 37°C. (Concentrations given in Table 2.) SP was added (10 "M) and activity was measured by bioassay on guinea pig ileum.

did not block the inactivation of SP by endothelial cells. DFP, an inhibitor of serine proteases, and inhibitors of trypsin-like enzymes, such as Trasylol " (aprotinin) and soybean trypsin inhibitor blocked the inactivation of SP. Alpha-N-tosylarginine methyl ester (TAMe), a substrate of trypsin, inhibited inactivation by 63% at a final concentration of 10^{-2}M. The dipeptide, leucylmethionylamide, was a more potent inhibitor because it inhibited at a final concentration of 10^{-4}M.

The inhibition pattern of the inactivation of the octapeptide was more complex. Both metal chelating agents, EDTA and o-phenanthrolene, as well as the protease inhibitors, soybean trypsin inhibitor, Trasylol ", and DFP prevented loss of spasmogenic action of SP_N during incubation with the endothelial cells (Table 3). The decline in activity on the guinea pig

TABLE 3. *Inhibition of the enzyme(s) in endothelial cells that inactivate the octapeptide (SP_N)*

Inhibitor	Conc.	Substrate	No.	% Inhibition
Soybean trypsin	600 μg/ml	Octapeptide (10^{-6}M)	2	58
Trasylol "	1,000 u/ml	"	4	75
EDTA	5×10^{-3}M	"	2	61
o-phenanthrolene	10^{-3}M	"	5	64
DFP	10^{-3}M	"	4	68

Cells were suspended, incubated with inhibitors for 15 min at 37°C prior to addition of substrate. Assayed on guinea pig ileum.

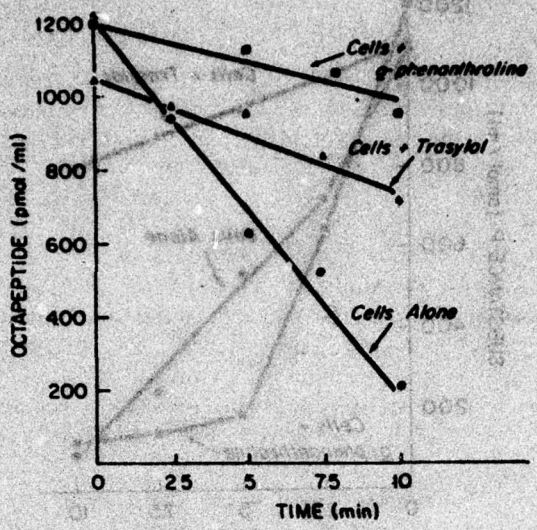

FIG. 3. Inactivation of SP_n by endothelial cells. Suspended cells (1.5×10^5) were incubated with buffer alone or with inhibitors (concentrations given in Table 3) for 15 min at 37°C. SP_n was added (10^{-8}M) and its activity was measured by bioassay on guinea pig ileum.

ileum during incubation with cells or cells and two different inhibitors is shown in Fig. 3.

DISCUSSION

Although SP is among the most active endogenous peptides, its enzymatic metabolism has not been investigated in detail. Pernow (14) indicated that chymotrypsin inactivates SP much faster than trypsin does when inactivation was measured by bioassay on the guinea pig ileum. He also noted that extracts from many tissues contained proteolytic inactivators of SP. Recently, Benuck and Marks (3) described a partially purified enzyme from rat brain that cleaved SP at two internal bonds and inactivated the peptide with respect to smooth muscle stimulating action.

In another study of enzymes in cultured human endothelial cells, we found that either monolayers or suspended cells could cleave vasoactive peptides such as angiotensin I and bradykinin. This activity was attributed to the angiotensin I converting enzyme, or kininase II (9,10). However, the enzyme was much more active when the cells were suspended than when they were in a monolayer. This increase in activity with suspension may be due to an increase in the surface area of the suspended cells and to an increase in the rate of interaction between substrate and the surface-bound enzyme.

We found that cultured human endothelial cells also inactivate SP, and that a similar increase in activity occurred when the cells were suspended from monolayers into separated cells. Although endothelial cells treated

with ^{125}I-labeled trypsin took up the label, inactivation of SP cannot be due to contamination with exogenous trypsin. First, cells in monolayers were able to inactivate SP and SP_n. Second, the cells suspended by scraping were as active as trypsin-suspended cells in cleavage of SP. Finally, trypsin treatment of scraped cells did not enhance the inactivation of SP. This observation supports reports of others (4,14) that SP is resistant to degradation by trypsin.

The initial site of cleavage has not been determined. If the endothelial enzyme is a tryptic enzyme similar to plasmin or thrombin, it might attack the Lys^3-Pro^4 bond and release SP_n which, under our conditions of assay, had less smooth muscle-stimulating activity than SP. However, the protease might cleave another peptide bond that is not adjacent to a basic amino acid. This was shown in experiments in which hydrolysis of SP_n was partially inhibited by protease inhibitors. Anastasi et al (1) observed that trypsin cleaves anomalously such a bond in physalaemin, a peptide that is structurally similar to SP.

Possibly, the enzyme that cleaves SP and SP_n is a peptidase with a heavy metal cofactor, since it is inhibited both by *o*-phenanthroline and by EDTA. This enzyme might attack SP_n at the N-terminal end and cleave the Pro^4-Glu^5 bond. These speculations will have to be confirmed by chemical assay of the products cleaved from the peptides, since only very limited information can be obtained from bioassay.

The importance of cleavage of SP by an enzyme bound to the surface of endothelial cells seems obvious. SP is a very potent hypotensive agent, and its release into blood would bring it into immediate contact with the vascular endothelium. Since the vasodilator actions of SP varies according to the particular vascular bed and the animal species tested (*this volume*), these differences may reflect, in part, differences in rates of inactivation of the peptide. Although peptide fragments of SP have biological activities (*this volume*), some actions may require the intact undecapeptide. For example, the sialogogue action (11,15) and the release of histamine from mast cells (8) are two of these. Histamine release by SP presumably depends upon the presence of basic amino acids in the peptide sequence. Conversion of SP to the octapeptide, SP_n by removal of the N-terminal tripeptide removes both the arginine and lysine that convey a net positive charge to the undecapeptide. Thus, SP_n might have a different action on various target cells. The octapeptide can be degraded by enzymes in endothelium and other tissues, and its actions would probably be brief and limited to a specific environment, such as the vascular smooth muscle of selected vascular beds.

We cannot delineate the actions of SP and its metabolic products *in vivo*. However, our experiments show that human endothelial cells grown in tissue culture can inactivate both SP and the octapeptide derivative, SP_n, and suggest that inactivation by endothelium contributes to the termination of the action of SP *in vivo*.

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